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Protocol Note

A novel method of genomic DNA extraction for Cactaceae[1]

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- *Premise of the study:* Genetic studies of Cactaceae can at times be impeded by difficult sampling logistics and/or high mucilage content in tissues. Simplifying sampling and DNA isolation through the use of cactus spines has not previously been investigated.
- *Methods and Results:* Several protocols for extracting DNA from spines were tested and modified to maximize yield, amplification, and sequencing. Sampling of and extraction from spines resulted in a simplified protocol overall and complete avoidance of mucilage as compared to typical tissue extractions. Sequences from one nuclear and three plastid regions were obtained across eight genera and 20 species of cacti using DNA extracted from spines.
- *Conclusions:* Genomic DNA useful for amplification and sequencing can be obtained from cactus spines. The protocols described here are valuable for any cactus species, but are particularly useful for investigators interested in sampling living collections, extensive field sampling, and/or conservation genetic studies.

Key words: cactus; cactus spines; DNA amplification; DNA extraction; DNA sequencing.

DNA sequencing techniques have been used to study cactus systematics for almost two decades (e.g., Wallace, 1995). Genomic DNA for these studies has routinely been obtained from various cactus tissues using both modified cetyltrimethylammonium bromide (CTAB) and mini kit extraction protocols (e.g., Arias et al., 2003; Hernández-Hernández et al., 2011; Franck et al., 2012; Majure et al., 2012). However, a number of investigators have commented on the difficulty of extracting genomic DNA from cactus tissues because of the high polysaccharide-based mucilage content and other interfering secondary compounds (e.g., de la Cruz et al., 1997; Mondragon-Jacobo et al., 2000; Nyffeler, 2002; Griffith and Porter, 2003; Edwards et al., 2005; Korotkova et al., 2011). Field investigations involving extensive population-level sampling and DNA sequencing of cacti are rare, possibly due to the difficult logistics of tissue collection and/or the conservation status of many cacti species. Tissues such as cladode leaves or flowers can be a good option for sampling (Majure et al., 2012); however, because these tissues are only available seasonally or in certain genera, sampling of epidermal tissue is more common. Sampling epidermal tissue from cactus stems can result in damage to the plants and exposure of the soft tissue to pathogens. Such tissue sampling is also time consuming and potentially hazardous to the investigator. Furthermore, when cacti of research interest are species of conservation concern or part of a living collection, it may be difficult to obtain permission to sample tissues (including epidermal, floral, or fruit tissues).

In seeking an alternative to sampling cactus tissues for DNA extraction, we reasoned that problems might be reduced or eliminated if DNA could be extracted from cactus spines. Spines are considered modified leaves (bud scales) and are composed of simplified cells found in three regions: a basal meristem, elongation zone, and apical zone of primarily dead cells (Mauseth, 2006). Spines lack the large amounts of mucilage found in other tissues, are easily removed without damaging the plant or investigator, require little storage space or special storage conditions, and are easily transported. In preparation for a conservation genetics study of the federally endangered Arizona hedgehog cactus, *Echinocereus arizonicus* Rose ex Orcutt subsp. *arizonicus* (Baker, 2006), we developed a protocol for DNA extraction from cactus spines and tested it across 20 species in eight genera. Here we demonstrate that genomic DNA can be isolated from cactus spines, and specific nuclear and plastid DNA regions can be amplified and successfully sequenced. In comparison to existing tissue sampling and extraction techniques, spine sampling and extraction is logistically and technically simpler. The protocols described here are useful for investigators interested in the phylogenetics, population, and/or conservation genetics of cacti.

METHODS AND RESULTS

Spines were harvested from eight genera in the living collection at the Desert Botanical Garden in Phoenix, Arizona, USA, and accession and/or voucher numbers were recorded where possible (Appendix 1; *Coryphantha*, *Denmoza*, *Echinocereus*, *Ferocactus*, *Mammillaria*, *Opuntia*, *Pachycereus*, and *Stenocereus*). We used a pair of jewelry-making bent-nose pliers and fine wire cutters to

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reach into tight places, grasp spines, and clip them at their base. Our investigations indicated that any spine and any part of the spine could be used, but fresh spines found at the apex of growing stems produced the highest DNA yields. Between five and 15 spines from each sampled individual were placed in 1.5-mL microtubes with or without silica gel and later stored at −20°C. Initial results with field-collected spines indicated that surface contaminants (such as pollen) could interfere with downstream results. Therefore, prior to extraction, we added a wash solution (10% bleach, 1% tween) to the spines in the 1.5-mL microtubes, vortexed, decanted, and rinsed twice with purified water to remove potential contaminants. For tissue disruption with a shaker (described below), results were improved when washed spines were dried at 65°C for several hours prior to shaking.

Genomic DNA was extracted using a DNeasy Plant Mini Kit (QIAGEN, Valencia, California, USA). The manufacturer's protocol was followed with some modifications for lysis and elution (Appendix 2). For maximum recovery of DNA, from 40 to 100 mg of starting material was used. Several methods of tissue disruption were tested, and although DNA yield varied with disruption technique, all methods resulted in successful extractions. Here we recommend two options for tissue disruption. (1) Spines were cut into smaller pieces using wire cutters or cuticle/curved nail scissors that could reach the bottom of a 1.5-mL microtube, and lysis buffer was added directly to the tubes. After an initial incubation of 30 min at 65°C, the softened spine material was disrupted by hand using a disposable micropestle. The material was disrupted a second time after an additional 30-min incubation in the lysis buffer, followed by a final incubation for 10 min. For some extraction trials, spines were left in lysis buffer overnight with similar results to the incubation described above. To concentrate the recovered DNA, 50 µL of elution buffer followed by an additional 50 µL or 75 µL were used in the final step. From 3 to 25 ng/µL of DNA were routinely recovered as measured by a Nanodrop Fluorospectrometer (Thermo Scientific, Wilmington, Delaware, USA). It is important to note that yields for some samples were too low to be detected by spectrometry and/or were not visible in agarose gels; however, these samples still resulted in successful amplification and sequencing. (2) Alternatively, dried spines were placed in 25-mL stainless steel grinding jars with 15-mm stainless steel balls and shaken at a frequency of 25 Hz for 60 s in a Mixer Mill MM 200 (Retsch, Newtown, Pennsylvania, USA). Extra care was taken to clean and sterilize the grinding jars and balls between samples. Powdered spines were transferred to a 1.5-mL microtube, and lysis buffer was added. Tubes were incubated at 65°C for 120 min with vortexing and inversion every 30 min. DNA was eluted with 75 µL of elution buffer, and the resulting elute was used again for the second elution. From 5 to 35 ng/µL of DNA were routinely recovered as measured by a Nanodrop Fluorospectrometer (but see note above).

Three noncoding chloroplast regions (*rpl16*, *trnL-trnF*, *psbA-trnH*) and one nuclear region (*ppc*) were used as exemplars. Primers used for amplification and sequencing are listed in Table 1. Numerous trials with several published PCR protocols (Arias et al., 2003; Butterworth and Wallace, 2004; Hernández-Hernández et al., 2011; Korotkova et al., 2011) resulted in mixed success. With the exception of the *trnL-trnF* region, only about 50% of the samples amplified in any given trial, and amplification and sequencing results were variable from trial to trial. Eventually we were introduced to KAPA2G Robust HotStart ReadyMix polymerase (Kapa Biosystems, Woburn, Massachusetts, USA), and amplification and sequencing consistency and success dramatically improved. The PCR protocols shown in Table 2 gave the most consistent results across DNA regions and genera. After the use of KAPA2G polymerase, amplification of the exemplar regions was obtained for representatives from all genera tested. Selected amplification products were purified by adding 0.025 µL of exonuclease I and 0.25 µL of shrimp alkaline phosphatase (Affymetrix, Santa Clara, California, USA) and incubating at 37°C for 30 min followed by 95°C for 5 min. Sequences were obtained using 1/16 BigDye Terminator version 3.1 (Life Technologies, Grand Island, New York, USA) cycle sequencing reactions. Thermocycling conditions were 94°C for 1 min, and 25 cycles of 94°C for 10 s, 50°C for 5 s, and 60°C for 30 s. Sequencing products were purified and visualized on an ABI 3130xl genetic analyzer at the Arizona State University DNA sequencing facility. For sequencing, the forward primer was used in most cases; the reverse primer was used in cases where more sequence clarity was needed. GenBank accession numbers for acquired sequencing regions are provided in Appendix 1. No editing or very little editing was required for sequences generated for *trnL-trnF* and *psbA-trnH*; however, the *rpl16* and *ppc* regions required more editing. Samples showing double amplification bands for a given region were not sequenced.

Table 1. DNA regions and primers used in this study.

Region	Primer name: sequence or reference
trnL-trnF	e: Taberlet et al., 1991
	f: Taberlet et al., 1991
psbA-trnH	psbA: Butterworth and Wallace, 2004
	trnH: Sang et al., 1997
	psbA3u: 5′-GCTAACCTTGGTATGGAAGT-3′
	trnHu: 5′-GGATTCACAATCCACTGCC-3′
rpl16	rpL16F71: Shaw et al., 2005
	rpL16R1516: Shaw et al., 2005
	rpl161f: Hernández-Hernández et al., 2011
	rpl163r: Hernández-Hernández et al., 2011
ppc	PPCX4F: Olson, 2002
	PPCX5R: Olson, 2002

We applied the protocols described here to spine and stem samples from herbarium specimens housed at the Desert Botanical Garden Herbarium (DES) with mixed success. In preliminary trials, we were able to obtain clean sequences for the *trnL-trnF* region in some cases; however, for most samples, a problem with contamination of the herbarium specimens with exogenous DNA was encountered. Additional technical work would need to be accomplished if this protocol is to be applied to herbarium specimens. However, others have had success with DNA extraction from stem or leaf tissue from herbarium specimens (e.g., Ocampo and Columbus, 2010; Majure et al., 2012).

Feasibility and utility of this sampling and extraction method is being demonstrated through a conservation genetic study of *E. arizonicus* subsp. *arizonicus* in our laboratory. Twenty-one sites near Superior, Arizona, were visited based on data from environmental assessment reports, Arizona Natural Heritage Program element occurrence records, and the Southwest Environmental Information Network (SEINet; http://swbiodiversity.org/seinet/index.php); exact geographical coordinates are not listed due to the sensitive nature of this species. An average of 12 individual plants (spines) were sampled for genetic analysis at each of the 21 sites for a total of 209 samples. DNA was extracted from 172 samples using the protocol described here (including removal of surface contaminants by washing and stainless steel grinding/shaking). Genetic data from seven microsatellite loci for all samples and *psbA-trnH*, *trnL-trnF*, and *ppc* sequences for a subset of samples were obtained. Microsatellite loci were developed specifically for *E. arizonicus* subsp. *arizonicus* using Illumina paired-end genomic sequencing (see Castoe et al., 2012). After an initial screening of 58 potential microsatellite loci, seven were selected and amplified using 12.5-µL volume PCR reactions: 4 µL nuclease-free water, 2.5 µL Promega 5× PCR buffer, 1.5 µL of 25 mM $MgCl_2$, 1.0 µL of 10 mM dNTPs, 1.25 µL of 10× bovine serum albumin, 0.30 µL of 10 µM 5′-GTTT-3′ tagged primer, 0.05 µL of 10 µM 5′-CAGTCGGGCGTCATCA-3′ tagged primer, 0.25 µL of 10 µM 5′-CAGTCGGGCGTCATCA-3′ FAM-labeled primer, 0.15 µL GoTaq DNA Polymerase (5 U/µL; Promega Corporation, Madison, Wisconsin, USA), and 1.5 µL DNA template. Thermocycling conditions consisted of a touchdown protocol with an initial denaturation step of 5 min at 95°C followed by 20 cycles of 95°C for 30 s, 60°C for 30 s (decreased 0.5°C per cycle), and 72°C for 30 s; and 20 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 30 s. Preliminary results indicate that microsatellite fragments ranged in size from 140 to 500 bp across all loci and at least five microsatellite loci were amplified and scored for each of the 172 samples. It was necessary to repeat amplification and scoring for very few individuals, between 10 and 40 depending on the locus. Special attention was given to the possibility of cross-contamination, and positive and negative controls were included throughout. Preliminary data analyses revealed genetic diversity and structure that was biologically and geographically meaningful.

Compared to our previous experiences with population-level sampling of cactus stem tissue, sampling of cactus spines for this conservation genetic study required less time per sample, less space used for and simplification of sample storage, reduced time for cleaning of sampling tools between samples, and easier storage and transport of collected material in the field. Simplification of field collection for large numbers of samples and more readily granted permission to obtain samples are two of the greatest advantages of the protocol described here. Compared to existing techniques for tissue extraction and our experiences with those techniques, DNA extraction from spines required less time, involved fewer modifications to the manufacturer's protocol, prevented complications from tissue mucilage, and avoided difficult techniques and/or techniques involving more toxic chemicals (e.g., Korotkova et al., 2011; Franck et al., 2012). However, DNA extraction from spines did not provide an advantage with respect to DNA yield; higher yields are likely to be obtained from

TABLE 2. PCR reaction mixes (25 μL total) and thermocycler parameters (Mastercycler Pro, Eppendorf, Westbury, New York, USA) for four DNA regions amplified and sequenced in this study.

PCR protocol	*trnL-trnF*	*psbA-trnH*	*rpl16*	*ppc*
PCR mix				
Nuclease-free water	8 μL	5–7 μL	4–6 μL	5 μL
1× KAPA2G ReadyMix	12.5 μL	12.5 μL	12.5 μL	12.5 μL
Primer 1 [10 μM]	1.25 μL	1.25 μL	1.75 μL	1.25 μL
Primer 2 [10 μM]	1.25 μL	1.25 μL	1.75 μL	1.25 μL
DNA template	2 μL	3–5 μL	3–5 μL	5 μL
Thermocycler parameters				
Initial melt	95°C 1 min	95°C 2 min	95°C 2 min	95°C 2 min
Cycles	35	40	40	37
Melt	95°C 10 s	95°C 15 s	95°C 15 s	95°C 12 s
Annealing	60°C 10 s	55°C 15 s	55°C 15 s	57°C 12 s
Elongation	72°C 10 s	72°C 30 s	72°C 30 s	72°C 20 s

other tissues using modified CTAB techniques, and extractions using these protocols may be useful when larger amounts of DNA are needed (i.e., next-generation sequencing). In addition, because DNA yields from spines are low, extra care must be taken to reduce the potential amplification of contaminant DNA during PCR. Precautions such as the use of negative controls (blanks) and filter tips for PCR reactions are advised. Finally, the use of mini kits for extraction and high-quality DNA polymerase and filter tips for amplification increases the cost of this protocol significantly over CTAB extraction, which has clearly been used successfully to obtain high-quality, high-yield DNA in a number of studies (e.g., Korotkova et al., 2011; Majure et al., 2012).

CONCLUSIONS

We are convinced that genomic DNA can be isolated, amplified, and sequenced from spines in a variety of cactus species. While the use of other tissues and protocols may be more appropriate and/or cost effective, DNA extraction from spines is relatively straightforward and provides an important alternative to tissue sampling when a large number of samples or when noninvasive samples are needed. Important aspects of this protocol include starting with enough spine material, removing surface contaminants, sufficiently disrupting spines, and using a high-quality polymerase for PCR amplification. This approach is useful for those interested in sampling living collections, extensive field sampling, and population and conservation genetic studies of cacti.

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Appendix 1. Voucher information for taxa used and GenBank accession numbers for sequences obtained in this study.

Genus	Species	Voucher/identifier[a]	Accession no.: *trnL-trnF*, *psbA-trnH*, *rpl16*, and *ppc*
Coryphantha (Engelm.) Lem.	*C. vivipara* var. *deserti* (Engelm.) W. T. Marshall	DES A. Salywon 1878	—, KC196848, KC196812, KC196824
	C. vivipara (Nutt.) Britton & Rose	DES A. Salywon 1885	KC196813, KC196847, KC196809, KC196825
Denmoza Britton & Rose	*D. rhodacantha* (Salm-Dyck) Britton & Rose	DBG 1992 0303	JX977075, KC196839, KC196806, —
Echinocereus Engelm.	*E. coccineus* Engelm.	DES W. Hodgson 25506	KC196814, —, KC196803, KC196826
	E. engelmannii (Parry ex Engelm.) Lem.	DBG 2005 3157	KC196815, —, —, —
	E. stoloniferus W. T. Marshall	DBG s.n.	—, —, —, KC196827
	E. websterianus G. E. Linds.	DBG 1990 0675	KC196816, —, KC196807, KC196828
Ferocactus Britton & Rose	*F. cylindraceus* (Engelm.) Orcutt	DBG s.n.	KC196817, KC196846, KC196805, —
	F. fordii (Orcutt) Britton & Rose	DBG 1962 7066	KC106818, KC196845, KC196810, —
Mammillaria Haw.	*M. grahamii* Engelm.	DBG 2009 0070	—, KC196844, KC196811, KC196829
	M. tetrancistra Engelm.	DBG s.n.	JX977076, KC196840, KC196805, —
	M. thornberi Orcutt	DBG s.n.	—, KC196843, KC196808, KC196830
Opuntia Mill.	*O. phaeacantha* Engelm.	DBG s.n.	—, KC196838, —, KC106831
	O. polyacantha Haw.	DBG s.n.	—, —, —, KC196832
Pachycereus (A. Berger) Britton & Rose	*P. hollianus* Buxb.	DBG 1974 0175	KC196819, —, —, KC196833
	P. pringlei (S. Watson) Britton & Rose	DBG s.n.	KC196820, —, —, KC196834
	P. sp.	DBG 1990 0759	KC196821, KC196842, —, KC195835
Stenocereus (A. Berger) Riccob.	*S. eruca* (Brandegee) A. C. Gibson & K. E. Horak	DBG s.n.	—, —, —, KC196836
	S. griseus (Haw.) Buxb.	DBG 1990 0828	KC196822, —, —, KC196837
	S. queretaroensis (F. A. C. Weber) Buxb.	DBG 1986 0554	KC196823, KC196841, —, —

[a] DES = vouchers deposited at Desert Botanical Garden Herbarium; DBG = plants part of the Desert Botanical Garden Living Collection.

Appendix 2. Generalized protocol for DNA extraction from cactus spines.

Note: Follow the manufacturer's mini protocol for the DNeasy Plant Mini Kit (QIAGEN, Valencia, California, USA) with the following adaptations.

1. Harvest five to 15 spines per plant (40–100 µg) using jewelry-making bent-nose pliers and fine wire cutters. Store spines in 1.5-mL microtubes with or without silica gel at −20°C.

2. To clean spine surfaces prior to extraction, cover spines in 1.5-mL microtubes with a 10% bleach, 1% tween solution and vortex. Decant the wash solution and rinse two times with purified water (add water, vortex, decant). Dry spines at 65°C for two to four hours.

3. Transfer spines to a 25-mL stainless steel jar with 15-mm stainless steel balls and shake at a frequency of 25 Hz for 60 s in a Mixer Mill MM 200 (Retsch, Newtown, Pennsylvania, USA). Clean and sterilize jars and balls between samples.

4. Transfer spine powder to a 1.5-mL microtube and add 400 µL of AP1 lysis buffer and 4 µL of RNase A, as directed in the manufacturer's mini protocol. Incubate at 65°C for 120 min, vortexing every 30 min.

5. Continue following the manufacturer's mini protocol. In the final elution step, if higher concentrations of DNA are desired, consider eluting with <100 µL of elution buffer and/or use the first elute for the second elution.